



PHL1 of *Cercospora zeae-maydis* encodes a member of the photolyase/cryptochrome family involved in UV protection and fungal development ☆

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ABSTRACT

DNA photolyases harvest light energy to repair genomic lesions induced by UV irradiation, whereas cryptochromes, presumptive descendants of 6–4 DNA photolyases, have evolved in plants and animals as blue-light photoreceptors that function exclusively in signal transduction. Orthologs of 6–4 photolyases are predicted to exist in the genomes of some filamentous fungi, but their function is unknown. In this study, we identified two putative photolyase-encoding genes in the maize foliar pathogen *Cercospora zeae-maydis*: *CPD1*, an ortholog of cyclobutane pyrimidine dimer (CPD) photolyases described in other filamentous fungi, and *PHL1*, a cryptochrome/6–4 photolyase-like gene. Strains disrupted in *PHL1* ($\Delta phl1$) displayed abnormalities in development and secondary metabolism but were unaffected in their ability to infect maize leaves. After exposure to lethal doses of UV light, conidia of $\Delta phl1$ strains were abolished in photoreactivation and displayed reduced expression of *CPD1*, as well as *RAD2* and *RVB2*, orthologs of genes involved in nucleotide excision and chromatin remodeling during DNA damage repair. This study presents the first characterization of a 6–4 photolyase ortholog in a filamentous fungus and provides evidence that *PHL1* regulates responses to UV irradiation.

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1. Introduction

As sessile organisms, fungi have developed highly effective strategies to cope with a variety of harmful environmental conditions, including excessive exposure to UV irradiation. UV light induces chromosomal damage, such as the formation of cyclobutane pyrimidine dimers (CPDs) and 6–4 pyrimidine-pyrimidone lesions (6–4 PPs) (Sinha and Hader, 2002), with the formation of CPD lesions predominating over 6–4 PP lesions at a ratio of approximately 10:1 (Douki and Cadet, 2001). UV-induced lesions are repaired by DNA photolyases, specialized enzymes that bind specifically to damaged DNA and harvest light energy to either break the cyclobutane ring formed in CPD lesions or to reverse the photoaddition of the C4 carbonyl of a thymine to the C5–C6 double bond of a neighboring pyrimidine in a 6–4 PP lesion (Weber, 2005). Two distinct classes of photolyases repair UV-induced lesions: the evolutionarily ancient CPD photolyases found in all tax-

onomic kingdoms, and the 6–4 photolyases, found only in multicellular Eukaryotes (Essen and Klar, 2006). Among plants and animals, homologs of 6–4 photolyases have evolved to form the cryptochrome family of blue-light photoreceptors, members of which regulate a wide variety of biological processes in response to light. Intriguingly, genes predicted to encode proteins homologous to 6–4 photolyases have been identified in the sequenced genomes of several filamentous fungi. Because no photolyase/cryptochrome-like gene has been characterized in fungi, it is unclear whether these genes function as DNA-repair enzymes or photoreceptors in the Fungal kingdom.

The anamorphic fungal genus *Cercospora* is comprised of numerous and diverse plant pathogens that affect important crops throughout the world. Many species of *Cercospora* produce the host non-specific phytotoxin cercosporin, a photosensitizing perylenequinone whose biosynthesis and activation are induced by visible light (Daub and Ehrenschaft, 2000). Recently, a cluster of genes encoding the enzymes required for cercosporin biosynthesis was identified and characterized (Chen et al., 2007), but little is known at the molecular level about the regulation of cercosporin biosynthesis by light. Presumably, the transcriptional activation of genes involved in cercosporin biosynthesis requires the function of a sensory protein such as a photoreceptor.

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the products, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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The focus of this study was to identify and characterize a cryptochrome/6–4 photolyase-like gene in the maize foliar pathogen *Cercospora zeae-maydis*. We cloned and disrupted *PHL1*, a gene highly homologous to photolyase- and cryptochrome-encoding genes from plants and animals. Disruption of *PHL1* completely abolished photoreactivation and impaired the expression of *CPD1*, a second putative photolyase identified in this study, as well as at least two other genes involved in DNA damage repair. Disruption of *PHL1* caused abnormalities in development and secondary metabolism, suggesting additional signaling functions for *PHL1*. We hypothesize that the fungal family of photolyase/cryptochrome-like genes such as *PHL1* have evolved regulatory functions that distinguish them from 6–4 photolyases in higher plants and animals.

2. Materials and methods

2.1. Fungal strains and growth conditions

Strain SCOH1-5 of *Cercospora zeae-maydis* was used as the wild type in this study. Strains $\Delta phl1-1$ and $\Delta phl1-2$ (disrupted in *PHL1*) were constructed as described below. Strains WT-GUS and *phl1*-GUS were constructed by transforming strains SCOH1-5 and strain $\Delta phl1-2$, respectively, with plasmid pDB23, a vector constructed by cloning a geneticin-resistance cassette from pKS-GEN (Bluhm et al., 2008), into the EcoRI site of vector pNOM102 (GenBank Accession No. Z32701). To promote conidiation, strains were maintained on V8 agar in constant darkness and transferred every 5 days. For analyses of cercosporin biosynthesis, fungi were grown on 0.2× potato dextrose agar (0.2× PDA; BD Biosciences, Sparks, MD). Tissue for genomic DNA was collected from cultures grown in liquid YEPA medium (0.5% yeast extract, 1.0% peptone, and 3% dextrose); tissue for RNA extractions was prepared by inoculating conidial suspensions onto cellophane membranes overlaid on either 0.2× PDA or V8-agar plates as described by Cooley et al., (2005). Light was provided by two General Electric (GE) F40CW-RS-MM (cool white) bulbs 16 in. above the plates. Light intensity at the plates was 15–17 $\mu\text{mol}/\text{m}^2/\text{s}$ as determined with a LiCor integrating spectrophotometer (model LI 188B). Experiments were monitored continuously to verify that exposure to light did not affect the temperature of the plates.

2.2. Nucleic acid manipulations

Plasmids were purified with the Wizard Plus SV Minipreps DNA purification system (Promega; Madison, WI). Fungal genomic DNA was extracted by a modified CTAB protocol (Proctor et al., 1997), and Southern analyses were performed following standard protocols (Sambrook and Russell, 2001). RNA was extracted with Trizol reagent (Invitrogen; Carlsbad, CA) and purified with an RNeasy miniprep purification kit (Qiagen; Valencia, CA). For analyses of gene expression, cDNA was generated with the Stratascript RT-PCR system (Stratagene) using random primers as template. DNA sequences were determined by the Purdue University Genomics Center (West Lafayette, IN).

2.3. Identification of *PHL1*, *CPD1*, *RAD2*, and *RVB1*

To identify *PHL1*, degenerate PCR primers *PHL1degF* (CKCATCTKGTTRTTGAGAAGGA) and *PHL1degR* (GTGCAGTSCAGCAYTGCCAGTT) were designed by aligning amino acid and nucleotide sequences of predicted genes from *Magnaporthe grisea*, *Fusarium graminearum*, *Aspergillus clavatus*, and *Ustilago maydis*. Genomic DNA of *C. zeae-maydis* was amplified with primers *PHL1degF* and *PHL1degR* to generate a 1.1-kb product that was

cloned into pGEM-T EZ (Promega) and sequenced. The remainder of the gene, designated *PHL1*, was obtained by genome-walker PCR (Clontech; Mountain View, CA). To walk upstream from the product obtained by degenerate PCR, we amplified genome-walker libraries first with primers *phl5p1* (AGCTTCGCCGGCCATGTGCATGACCT) and *phl5p2* (CAGGAGCATAAGCGTCCGTGCTCTT) to obtain 914 bp of the gene followed by amplification with primers *phl15p3* (TACAACAAGGCTCCCAACGCACGG) and *phl15p4* (ATGCGATGCGATGCGCTGCTGCTGCCGAT) to obtain 385 bp of the gene. Walking downstream from the original 1.1-kb product was accomplished first by amplifying libraries with primers *phl3p1* (AGCACTA GGCTGGCAATTCGGCCAAA) and *phl3p2* (CTTCATCCCTGGCACCTCCCTTCCAA) to obtain 684 bp of *PHL1* followed by amplification with primers *phl3p3* (TGATGGTGATGGGTCCGAGACGAAG) and *phl3p4* (GGATGGATTGGCGTTGTATCCGAAA) to obtain 1275 bp of the gene. In total, we sequenced 3895 bp of the *PHL1* locus, including the entire open reading frame of *PHL1*, 837-bp upstream from the putative start codon, and 870-bp downstream from the putative stop codon. The entire sequence of the *PHL1* locus was deposited in GenBank (Accession No. EU443730).

To identify *CPD1*, degenerate PCR primers *CPDdegF* (GAATNGCMTGGCGRGARTTTTAC) and *CPDdegR* (ATCATRCGNAGNCGGTTGTGCAT) were designed by aligning amino acid sequences of CPD photolyases from *Bipolaris oryzae* (*PHR1*; AB126091) and *Neurospora crassa* (*phr*; X58713) as well as predicted genes from *Mycosphaerella graminicola*, *Magnaporthe grisea*, *Fusarium graminearum*, and *Aspergillus clavatus*. A 219-bp product amplified from genomic DNA of *C. zeae-maydis* with primers *CPDdegF* and *CPDdegR* was cloned into pGEM-T-EZ, sequenced, and submitted to GenBank (Accession No. EU814871).

To identify orthologs of other genes involved in DNA repair, we performed homology-based searches of proteins predicted to be encoded by 27,551 ESTs generated from *C. zeae-maydis* (Bluhm et al., unpublished). Cluster consensus sequence 496_1 (GenBank Accession No. EU443731), a 903-bp sequence obtained by analyzing three overlapping ESTs from two distinct clones, was highly similar to *RAD2* from *Aspergillus clavatus* (XM_001267902). Singlet EST 466_0 (GenBank Accession No. EU443732), a 723-bp sequence, was predicted to encode a protein highly similar to Ruv-B of *Escherichia coli* (AAA24613).

2.4. Disruption of *PHL1*

For functional analysis of *PHL1*, we targeted the gene for disruption via single homologous recombination. To build the disruption construct, 535 bp of the open reading frame was amplified with primers *PHL1F1* (GGCGAGATCGAAGAGCCTGTTG) and *PHL1F2* (TTTGCCGAATTGCCAGCTA) and cloned into pGEM-T EZ to create pDB68. Then, a *NotI* fragment from pDB68 was cloned into pKS-HYG (Bluhm and Woloshuk, 2005) to create pDB71. For transformation, a 2.0-kb product containing a 1.4-kb hygromycin-resistance cassette and 535 bp of *PHL1* sequence was amplified from pDB71 with primers *PHL1F1* and *HYG-F* (GATATTGAAGGAGCATTTTTTGGGCT). Protoplasts of *C. zeae-maydis* were prepared and transformed as described by Shim and Dunkle (2003). Hygromycin-resistant colonies were screened by PCR with primers A1 (CATTTGGTGTGTTGAGAAGGACACGGA) and H3 (CGGCAATTCGATGATGCAGCTTG) to identify two independent strains disrupted in *PHL1* ($\Delta phl1-1$ and $\Delta phl1-2$). For Southern analysis, genomic DNA was probed with the hygromycin-resistance cassette from pKS-HYG.

2.5. Quantification of cercosporin biosynthesis

Freshly harvested conidia of each strain were inoculated onto 0.2× PDA plates and incubated at 22 °C under either constant light

or darkness. After 4 days of growth, plates were flooded with 5 N KOH (5 ml) and incubated at room temperature for 30 min. Cercosporin concentrations were determined by measuring the A_{480} of each extract with a Beckman DU-530 spectrophotometer (Beckman Coulter; Fullerton, CA) and applying the extinction coefficient of 23,300 (Yamazaki and Ogawa, 1972). Experiments were repeated five times with similar results.

2.6. Analysis of gene expression

Expression of *PHL1* (forward primer AGTTCTGGGATTGCTG GACCGAAA, reverse primer TCTCGCCACCTTTATGAGCGAA), *CPD1* (forward primer CTCGAATAGAGCATCGTCTATTCCC, reverse primer TGGCATGGCGGGAGTTTACAAG), *RAD2* (forward primer ACT GGTCCATCATACTCGCTTCCT, reverse primer GATCAATTTC TCCA TCCTCGTCAGCT), and *RVB2* (forward primer AGACCTGGCCTGT AGTCCAAG, reverse primer ACCATGGCTGCACCCATCA), was measured by quantitative PCR. Reactions were performed in an MXP-3000 real-time PCR system (Stratagene; La Jolla, CA). Each reaction contained 10 μ l of QuantiTect SYBR[®]-green PCR Master mix (Qiagen), forward and reverse primers (500 nM of each), cDNA template, and nuclease-free water to a final volume of 20 μ l. PCR

cycling conditions were 10 min at 95 °C (1 cycle), 15 s at 95 °C followed by 1 min at 60 °C (40 cycles), and a melting curve of 1 min at 95 °C followed by 30 s at 55 °C and a final ramp to 95 °C with continuous data collection (1 cycle) to test for primer dimers and non-specific amplification. Expression of genes was measured in triplicate. To normalize expression data, 18S rRNA (GenBank Accession No. EU399178) was amplified with primers 18SrtF (CAGGC CTTTGCTCGAATACATTAGCAT) and 18SrtR (GGATGCCCCGAC TATCCCTATTA). To verify that the efficiencies of the target and reference reactions were approximately equal, reactions were performed using the primers for each gene or 18S rRNA with serial dilutions of cDNA as template. After verifying that the efficiencies of the primers were acceptable, expression levels were calculated by the comparative C_t method (Applied Biosystems) with 18S rRNA as the endogenous reference for normalization. The experiment was performed twice with similar results.

2.7. Determination of UV sensitivity and photoreactivation

For each strain, 5 μ l of water containing 5000, 1000, 500, or 100 conidia were spotted onto V8-agar plates which were exposed to UV light (10 mW/cm²) for 30 min. After irradiation, plates were

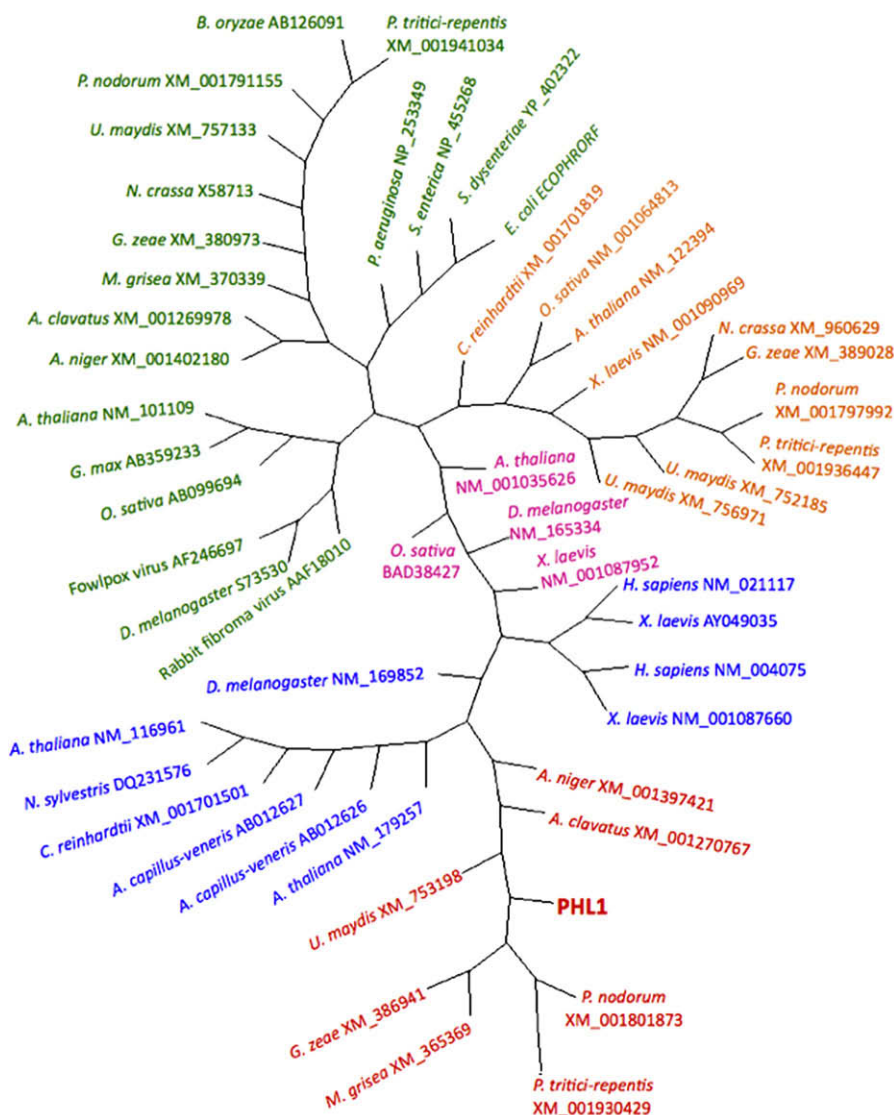


Fig. 1. Unrooted phylogenetic tree comparing amino acid sequences from fungal cryptochrome/6-4 photolyase orthologs (red font), CRY-DASH cryptochromes (orange font), plant and animal cryptochromes (blue font), CRY-DASH cryptochromes (orange font), plant and animal 6-4 photolyases (lavender font), and CPD photolyases (green font). Sequence alignments were performed with ClustalW (Larkin et al., 2007) and the tree was constructed with PHYLIP (Felsenstein, 2008).

incubated for 3 days in either constant light as described above (to promote photoreactivation) or constant darkness. For a control, plates inoculated in an identical manner but without exposure to UV were incubated for three days in either constant light or constant darkness. In each experiment, at least two plates were evaluated for each treatment, and the experiment was repeated three times with similar results.

2.8. Pathogenicity assays

Maize inbred B73, which is highly susceptible to infection by *C. zeae-maydis*, was inoculated in a greenhouse when plants were at the V5 stage of development (approximately 5 weeks after germination). Leaves were inoculated a suspension of 10^5 conidia/ml (1 ml) applied with an atomizer attached to an air compressor. Inoculated plants were incubated under opaque plastic bags for 5 days to promote symptom development. In each experiment, at least three plants were inoculated with each strain. The pathogenicity assays were performed five times with similar results.

3. Results

3.1. *PHL1* of *C. zeae-maydis* encodes a protein homologous to 6-4 photolyases and cryptochromes

Genes encoding proteins highly similar to 6-4 photolyases and cryptochromes are present in the sequenced genomes of several filamentous fungi, including *Fusarium graminearum*, *Magnaporthe grisea*, *Aspergillus clavatus*, *Aspergillus niger*, and *Ustilago maydis*. To determine whether the genome of *C. zeae-maydis* contains an orthologous gene, we performed PCR with degenerate primers designed from highly conserved regions of the genes described above which yielded a 1.1-kb product; the remainder of the gene (designated *PHL1*) was obtained by genome-walker PCR. A conceptual translation of *PHL1* yields a protein of 691 amino acids highly similar to a group of proteins predicted from uncharacterized genes in a variety of filamentous fungi (red font; Fig. 1). A phylogenetic analysis of representative CPD photolyases, 6-4 photolyases, human and animal cryptochromes, and hypothetical proteins from a variety of filamentous fungi indicates that the proteins encoded by *PHL1* and orthologs in other fungi appear to be more similar

to plant and animal cryptochromes than 6-4 photolyases (Fig. 1). We followed a similar PCR-based strategy to identify a CPD photolyase, designated *CPD1*, in *C. zeae-maydis*. Because we obtained only part of the open reading frame of *CPD1*, we could not include it in the phylogenetic analysis, but homology-based searches indicated high levels of similarity to other fungal CPD photolyases, including *phr1* of *Bipolaris oryzae* and *phr* of *Neurospora crassa* (data not shown).

PHL1 is predicted to contain an open reading frame of 2188 bp interrupted by two short introns of 52- and 60-bp near the 5' end of the coding region (Fig. 2A). The region of the protein comprised by amino acids 4–184 is highly homologous to the light-harvesting cofactor domain of photolyases and cryptochromes (pfam 00875), and the region of the protein comprised by amino acids 239–577 is highly homologous to the FAD-binding domain of photolyases and cryptochromes (pfam 03441). Among the proteins encoded by fungal orthologs of *PHL1*, the C-termini (amino acids 578–691 for *phl1*) are not highly conserved (Fig. 2B). One of the only shared characteristics of this region is that the terminal residues frequently comprise a basic, lysine-rich tail (Fig. 2B). An alignment of fungal 6-4 photolyase-like proteins with cryptochromes and 6-

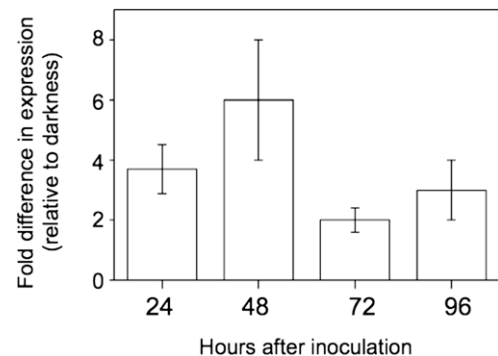


Fig. 3. Expression of *PHL1* is induced by light. Expression of *PHL1* was measured by qPCR in the wild-type strain 24, 48, 72, and 96 hr after inoculation onto 0.2x PDA medium in constant light or darkness. For each time point, expression of *PHL1* was normalized to 18S rRNA and is expressed relative to expression in darkness. Error bars represent the range as calculated by evaluating the expression $2^{-\Delta\Delta C_t}$ with $\Delta\Delta C_t + s$ and $\Delta\Delta C_t - s$, where s equals the standard deviation of the $\Delta\Delta C_t$ value.

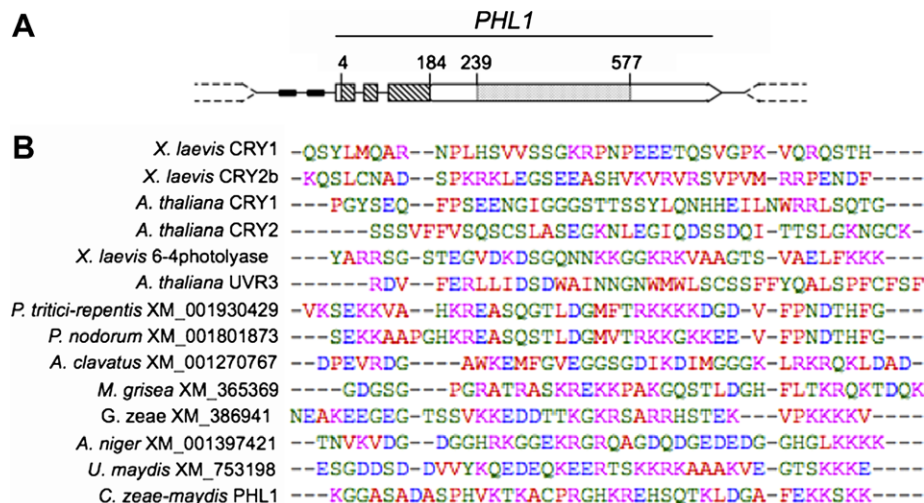


Fig. 2. *PHL1* of *C. zeae-maydis* encodes a protein belonging to the cryptochrome/6-4 photolyase family. (A) The open reading frame of *PHL1* is predicted to encode a protein of 641 amino acids with regions highly similar to the light-harvesting cofactor domain of photolyases and cryptochromes (hatched lines) and the FAD-binding domain of photolyases and cryptochromes (shaded). At least two putative GATA-derived regulatory elements are present in the promoter of the gene (black rectangles). (B) Alignment of the C-terminal region of the proteins encoded by *PHL1*, orthologous genes in other fungi, representative 6-4 photolyases, and representative cryptochromes.

4 photolyases from *Arabidopsis thaliana* and *Xenopus laevis* fails to distinguish the fungal proteins as belonging to one group or the other (Fig. 2B), although plant and animal cryptochromes generally lack the lysine-rich tail (data not shown). Although the function of this tail, if any, is not known, lysine-rich C-terminal tails have been implicated in mitogenic activities (Bernard-Pierrot et al., 2001) and protein–protein interactions (Pollock et al., 2004) in other families of proteins.

The regulation of gene expression by light occurs in fungi, at least in part, through the presence of GATA-derived regulatory elements in the promoter regions of light-responsive genes (He and Liu, 2005). Sequencing beyond the *PHL1* locus revealed the presence of neighboring coding regions flanking the *PHL1* open reading frame (Fig. 2A), indicating that the core promoter of the *PHL1* gene contains at most 620 nucleotides. Consistent with *PHL1* encoding a protein involved in light signaling responses and/or UV-induced DNA repair, at least two degenerate GATA sequences are present within the *PHL1* promoter (Fig. 2A).

3.2. Expression of *PHL1* is induced by light concurrent with the onset of cercosporin biosynthesis

In fungi, many CPD photolyases are transcriptionally regulated in response to visible light (e.g. Alejandro-Durán et al., 2003; Berrocal-Tito et al., 1999). However, expression of 6–4 photolyases is believed to be constitutive in plants and animals (Waterworth et al., 2002). We determined that the expression of *PHL1* is strongly induced by visible light irrespective of whether cultures were grown on media that are conducive or suppressive for cercosporin biosynthesis (Fig. 3). Furthermore, during growth on 0.2× PDA, a medium that supports high levels of cercosporin biosynthesis, expression of *PHL1* increased immediately prior to the onset of detectable cercosporin accumulation in the medium (Fig. 3). Consistent with the presence of putative light-responsive elements in the promoter of the gene, these results confirmed that expression of *PHL1* is induced by light and suggested a possible regulatory role for *PHL1* in cercosporin biosynthesis.

3.3. Disruption of *PHL1* affects conidiation and cercosporin biosynthesis

In order to characterize the role of *PHL1* in fungal growth and development, we targeted the gene for insertional disruption via homologous recombination (Fig. 4A). Of 96 hygromycin-resistant transformants screened by PCR, two tested positive for the insertion of a hygromycin-resistance cassette into the open reading frame of *PHL1* (Fig. 4B). Further analysis of the two independent mutants indicated that the disruption of *PHL1* resulted from an insertion of the hygromycin-resistance cassette into the open reading frame of the gene rather than a large-scale deletion or translocation at the *PHL1* locus, although insertion of the construct appears to have deleted approximately 200 additional base pairs in the $\Delta phl1$ -2 strain (Fig. 4B). Southern analysis of the two strains confirms that the $\Delta phl1$ -1 and $\Delta phl1$ -2 strains are both disrupted in *PHL1* (Fig. 4C). However, the presence of an additional band of approximately 6 kb in the $\Delta phl1$ -2 strain likely reflects an additional, ectopic insertion of the disruption cassette. (Fig. 4C). Despite the additional, ectopic insertion in the $\Delta phl1$ -2 strain, the two mutants were morphologically indistinguishable from each other when evaluated in a wide range of culture conditions (data not shown).

Other than nutritional requirements, the environmental conditions influencing conidiation in *C. zeae-maydis* are poorly defined. To determine the influence of light on conidiation, we counted the number of spores produced by the wild type strain during growth in constant light or darkness. During growth on V8 agar,

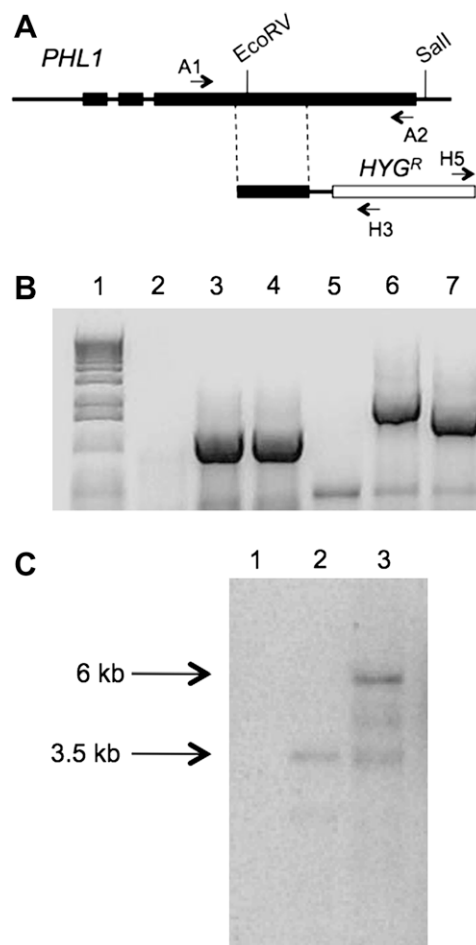


Fig. 4. Disruption of *PHL1*. (A) Disruption of *PHL1* was accomplished by inserting a hygromycin-resistance cassette (HYG^R) into the open reading frame of the gene via homologous recombination. (B) Identification of strains disrupted in *PHL1* ($\Delta phl1$) by PCR. A size standard (1 kb DNA ladder; Invitrogen) is shown in lane 1. Two transformants were identified with primers A1 and H3 as disrupted in *PHL1* (wild type, lane 2; $\Delta phl1$ -1, lane 3; $\Delta phl1$ -2, lane 4). Insertion of the HYG^R cassette into *PHL1* was verified with primers H5 and A2 (wild type, lane 5; $\Delta phl1$ -1, lane 6; $\Delta phl1$ -2, lane 7). (C) Southern blot analysis of the wild type (lane 1), $\Delta phl1$ -1 (lane 2), and $\Delta phl1$ -2 (lane 3) strains. Genomic DNA was digested with *EcoRV* and *SacI* and probed with the hygromycin-resistance cassette. In the wild-type *PHL1* locus, digestion with *EcoRV* and *SacI* would yield a 2089-bp fragment of DNA spanning the disruption site; insertion of the hygromycin-resistance cassette would be predicted to increase the size to approximately 3.5 kb. The presence of an additional hybridizing band at approximately 6 kb in the $\Delta phl1$ -2 strain is likely due to the ectopic insertion of an additional copy of the disruption cassette.

exposure to constant light resulted in dramatically reduced levels of conidiation, whereas high levels of conidiation were observed during growth in constant darkness. Both the $\Delta phl1$ -1 and $\Delta phl1$ -2 strains produced approximately five times more conidia than wild type in constant darkness (Fig. 5). However, disruption of *PHL1* did not promote conidiation during constant light (Fig. 5), suggesting that the repression of conidiation by constant light does not require the function of *PHL1*. These observations indicate that, although *PHL1* plays some role in regulating conidiation, it does not appear to encode a positive-acting photoreceptor required for the perception of light.

Because light induces cercosporin biosynthesis, we evaluated the ability of the $\Delta phl1$ -1 and $\Delta phl1$ -2 strains to produce cercosporin in culture. When grown in constant light, wild type cultures of *C. zeae-maydis* accumulate visible levels of cercosporin approximately 4 days after inoculation (Fig. 5). In strains $\Delta phl1$ -1 and $\Delta phl1$ -2, the onset of cercosporin accumulation occurred at the

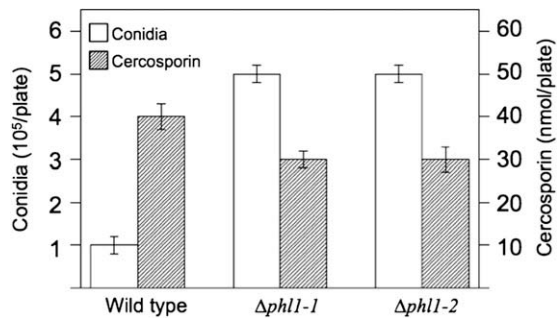


Fig. 5. Disruption of *PHL1* affects the production of conidia and cercosporin. Production of conidia in the wild type, $\Delta phl1-1$, and $\Delta phl1-2$ strains after four days of growth on V8 agar in constant darkness (left coordinate); cercosporin biosynthesis by the wild type, $\Delta phl1-1$, and $\Delta phl1-2$ strains after seven days of growth on 0.2× PDA (right coordinate).

same time as observed for the wild type, but was consistently less than the amount produced by the wild type strain (Fig. 5).

3.4. *PHL1* is dispensable for pathogenesis

Infection of maize leaves by *C. zeae-maydis* occurs after conidia deposited on leaves germinate and the emerging germ tubes form appressoria over stomata. After penetration through stomata, colonization of leaves by *C. zeae-maydis* results in distinctive rectangular lesions bounded by the major veins of leaves. To determine whether *PHL1* regulates aspects of development required for pathogenesis, we inoculated maize leaves with the wild type, $\Delta phl1-1$, and $\Delta phl1-2$ strains. All three strains aggressively infected maize leaves and caused expanding, necrotic lesions within 14 days after inoculation (data not shown). To evaluate fungal growth and development during pathogenesis, we inoculated maize leaves with the GUS reporter strains WT-GUS (wild type) and *PHL1*-GUS ($\Delta phl1-2$). Both strains produced multilobed appressoria over stomata within three days of inoculation (Fig. 6A and B), confirming that *PHL1* does not regulate stomatal tropism of germ tubes or

the formation of appressoria. By 12 days after inoculation, growth of both the wild type and $\Delta phl1$ reporter was comparable (Fig. 6C and D), resulting in the formation of necrotic lesions. From these findings, we conclude that *PHL1* does not play a significant role in infection or colonization of host tissue.

3.5. *PHL1* is required for photoreactivation and the induction of genes involved in repairing UV-induced DNA damage

Photoreactivation, the process in which photolyases utilize light energy to repair UV-damaged DNA, can be assessed by comparing survival after UV exposure between cultures allowed to recover in light versus darkness. The wild-type strain showed substantially greater survival after exposure to UV during recovery in constant light (Fig. 7A) compared to darkness (Fig. 7B), thus verifying that photoreactivation occurs in *C. zeae-maydis*. However, survival of the $\Delta phl1$ strains after UV irradiation was virtually identical regardless of exposure to light (Fig. 7A and B) and impossible to differentiate from wild type after recovery in darkness (Fig. 7B). This result indicates that photoreactivation is severely reduced, if not completely abolished, by disruption of *PHL1*.

Because 6–4 photoproducts constitute a relatively minor component of UV-induced DNA damage, the dramatic reduction in photoreactivation in the $\Delta phl1-1$ and $\Delta phl1-2$ strains suggested that *PHL1* regulates the expression or activity of *CPD1*. To test this possibility, we measured the expression of *CPD1* in wild type, $\Delta phl1-1$, and $\Delta phl1-2$ cultures incubated in light and darkness after UV irradiation. Before exposure to UV, the basal expression of *CPD1* was substantially lower in both the $\Delta phl1-1$ and $\Delta phl1-2$ strains (Table 1). After 30 min of photoreactivation, expression of *CPD1* was slightly higher in the wild-type strain, whereas no induction was observed in either the $\Delta phl1-1$ or $\Delta phl1-2$ strains (Table 1). This finding indicated that a functional copy of *PHL1* is necessary for wild-type levels of *CPD1* expression.

In addition to the activities of photolyases, UV-induced DNA damage is repaired by the activation of highly conserved signal transduction networks, which in turn regulate repair mechanisms such as the nucleotide excision repair (NER) pathway. To deter-

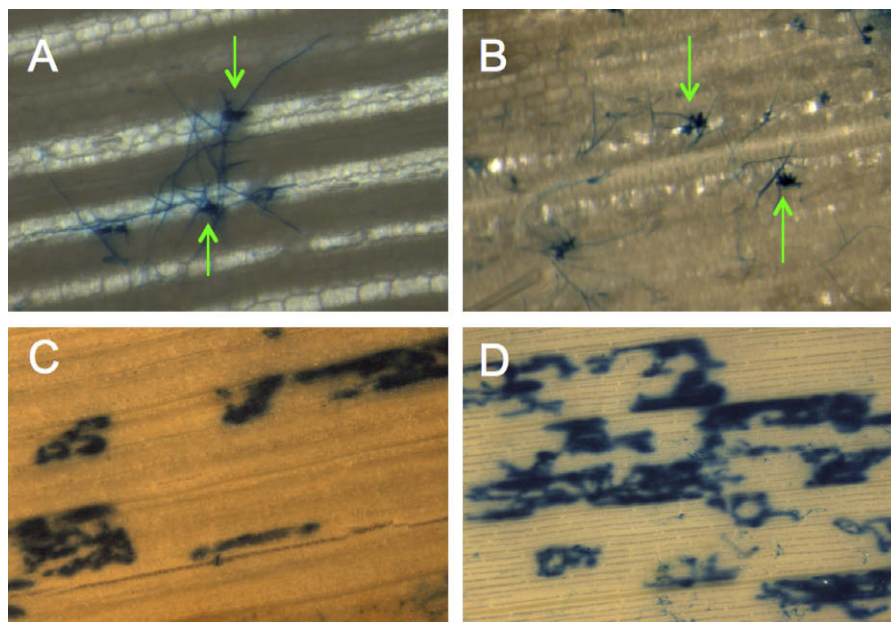


Fig. 6. *PHL1* is not required for infection or colonization of host tissue. Infection of maize leaves five days after inoculation with strain GUS-WT, wild-type *C. zeae-maydis* harboring a GUS reporter cassette (A), and strain *phl1-1*-GUS, the $\Delta phl1-1$ strain harboring a GUS reporter cassette (B). Appressoria are indicated with green arrows. By 14 days after infection, strain GUS-WT (C), and *phl1-1*-GUS (D) both colonize leaf tissue extensively, inducing expanding, necrotic lesions characteristic of gray leaf spot.

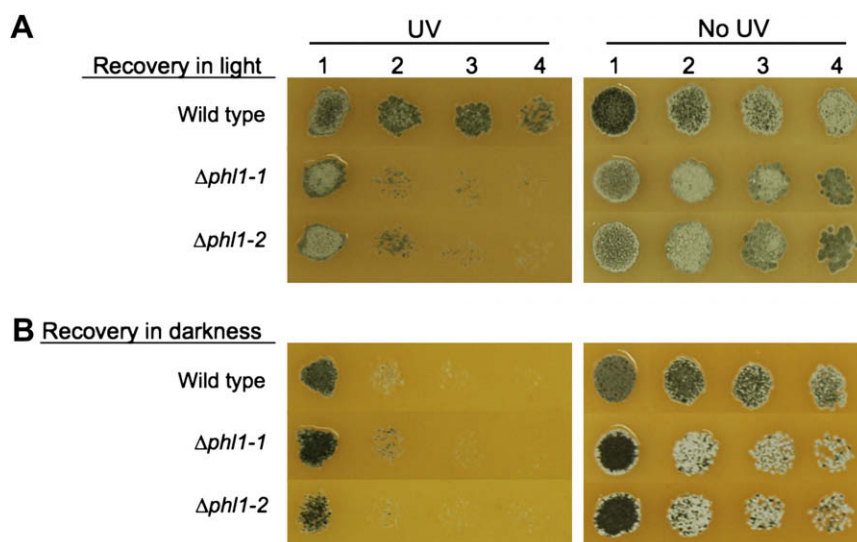


Fig. 7. Photoreactivation of the wild type, $\Delta phl1-1$, and $\Delta phl1-2$ strains after exposure to UV light. Dilutions of conidia from each strain were inoculated onto V8 agar [5×10^3 (column 1), 1×10^3 (column 2), 5×10^2 (column 3), and 1×10^2 (column 4)] were exposed to UV light for 30 min and incubated in either constant light (A) or constant darkness (B) for three days.

Table 1
Regulation of genes involved in DNA damage repair by *PHL1*^a

Gene	Before UV			After photoreactivation ^b		
	WT	$\Delta phl1-1$	$\Delta phl1-2$	WT	$\Delta phl1-1$	$\Delta phl1-2$
<i>CPD1</i>	1.0 (0.9–1.1)	0.3 (0.2–0.4)	0.7 (0.6–0.8)	1.5 (1.3–1.7)	0.4 (0.3–0.5)	0.8 (0.7–0.9)
<i>RAD2</i>	1.0 (0.9–1.1)	0.4 (0.3–0.5)	0.7 (0.5–0.8)	1.4 (1.3–1.5)	1.0 (0.8–1.2)	1.1 (1.0–1.2)
<i>RVB2</i>	1.0 (0.9–1.1)	0.5 (0.4–0.6)	0.8 (0.7–0.9)	2.1 (1.9–2.3)	0.6 (0.5–0.7)	1.0 (0.8–1.2)

^a For each gene, expression was normalized to 18S rRNA levels and calculated as fold differences in expression relative to expression in the wild type before exposure to UV light. Values in parentheses represent the range as calculated by evaluating the expression $2^{-\Delta\Delta C_t}$ with $\Delta\Delta C_t + s$ and $\Delta\Delta C_t - s$, where s equals the standard deviation of the $\Delta\Delta C_t$ value.

^b After exposure to UV light, cultures were incubated in white light for 30 min to promote photoreactivation.

mine the involvement of *PHL1* in regulating DNA damage repair, we searched an EST data set (Bluhm et al., unpublished) for orthologs of genes involved in various aspects of repair in *C. zeae-maydis*. We identified orthologs of *RAD2*, a gene encoding an endonuclease required for NER (Tomkinson et al., 1993) and *RVB2*, a gene encoding a protein required for the resolution of Holliday junctions formed during double-stranded break repair (West, 1997). To determine whether *PHL1* regulates either of these genes, we irradiated the wild type and mutant strains with UV and measured gene expression after recovery in constant light for 30 min. Consistent with observations that NER is not dependent on light energy (de Laat et al., 1999), expression of *RAD2* was not strongly induced by UV in the wild type, and transcript levels were only slightly lower in the $\Delta phl1$ strains (Table 1). In contrast, orthologs of *RVB2* are induced as much as fivefold by exposure to UV light (Hishida et al., 1999). In *C. zeae-maydis*, we found that the basal level of *RVB2* expression before UV irradiation was lower in the $\Delta phl1-1$ and $\Delta phl1-2$ strains, and the induction of the gene after exposure to UV was reduced in the Δphl mutants (Table 1). This finding combined with the dramatic reduction in photoreactivation in the $\Delta phl1-1$ and $\Delta phl1-2$ strains indicates that *PHL1* regulates at least some aspects of light-regulated DNA-repair mechanisms.

4. Discussion

Although both 6–4 DNA photolyases and homologous cryptochromes have been characterized extensively in plants and animals, the molecular functions of orthologous genes in fungi are not well defined. In this study, we demonstrate that disruption of

PHL1 in *C. zeae-maydis* results in developmental abnormalities, including increased levels of conidiation and reduced levels of cercosporin biosynthesis during growth in culture. Most significantly, disruption of *PHL1* completely abolishes photoreactivation after exposure to UV light, indicating that *PHL1* encodes either a photolyase, a regulator of photolyase activity, or a protein with both regulatory and DNA-repair activities. A comparative analysis of the proteins encoded by known photolyases and cryptochromes offers few clues as to the specific molecular function of the protein encoded by *PHL1*. Cryptochromes and 6–4 photolyases are highly similar throughout their light-harvesting and FAD-binding domains, with only a relatively small (typically < 100 amino acid) C-terminal region of the protein presumably being responsible for the specificity of molecular function. The phylogenetic analysis presented in this study suggests that the protein encoded by *PHL1* is more similar to plant and animal cryptochromes than 6–4 photolyases. However, *PHL1* and orthologous genes in other filamentous fungi encode proteins that share relatively low levels of homology in their C-termini with either cryptochromes or photolyases from plants and animals. In Arabidopsis, the C-termini of cryptochromes have been established as the signaling components of the proteins (Yang et al., 2000), and presumably the same is true for proteins encoded by orthologous genes in fungi. However, in order to fully understand the roles of proteins encoded by fungal cryptochrome/6–4 photolyase genes, functional analyses of C-terminal regions need to be performed.

Consistent with its homology to known photolyases, the protein encoded by *PHL1* is required for photoreactivation after exposure to UV light. However, at least two lines of evidence support the

conclusion that *PHL1* regulates broader aspects of DNA repair after exposure to UV. First, UV-induced lesions are predominantly CPD photoproducts, with 6–4 photoproducts comprising a comparatively minor class of DNA damage (Douki and Cadet, 2001). However, the molecular mechanism through which CPD expression is induced in response to UV is not known. In *Fusarium oxysporum*, the CPD photolyase encoded by *PHR1* plays a major role in photo-reactivation after UV irradiation, and expression of *PHR1* is induced by light through an unknown mechanism (Alejandre-Durán et al., 2003). *PHR1*, a gene from *Trichoderma atroviride* encoding a CPD photolyase, was recently demonstrated to regulate its own expression in a light-dependent manner (Berrocal-Tito et al., 2007), and genetic evidence indicates that its expression is also regulated by *blr-1* and *blr-2*, orthologs of *wc-1* and *wc-2* from *N. crassa* (Casas-Flores et al., 2004). Induction of *CPD1*, the CPD photolyase-encoding gene of *C. zeae-maydis* identified in this study, is impaired in the $\Delta phl1-1$ and $\Delta phl1-2$ strains, thus supporting the hypothesis that *PHL1* globally regulates photolyase-dependent DNA repair. Additionally, disruption of *PHL1* negatively affects the expression of at least two other genes involved in repairing DNA damage. Together, these observations indicate that *PHL1* encodes either a photoreceptor, a regulator of light-dependent gene expression, or a multi-functional enzyme involved in both DNA repair and signal transduction. Although cryptochromes in plants and animals possess signaling functions exclusively (Lin and Todo, 2005), members of the fungal cryptochrome/6–4 photolyase family have evolved separately from their plant and animal counterparts and could participate in novel signaling mechanisms. Alternatively, fungal members of the cryptochrome/6–4 photolyase family may represent evolutionary intermediates between UV-repair enzymes and photosensory regulators of gene expression. To resolve this uncertainty, future research will focus on identifying genes regulated by *PHL1* and defining molecular mechanisms through which *PHL1* functions in signaling pathways.

Although *PHL1* appears to play a role in the regulation of cercosporin biosynthesis, its exact function is not clear. The induction of cercosporin biosynthesis by light presumably requires the activity of an as-yet undiscovered photoreceptor. The induction of cercosporin biosynthesis is intact in the Δphl strains, suggesting that *PHL1* does not perform an inductive function or that the induction of cercosporin biosynthesis by light occurs through multiple and at least partially redundant signaling pathways. At least two scenarios can be proposed to explain this observation. One possibility is that the regulation of cercosporin biosynthesis by light results from complex interactions between multiple wavelengths of light. Among filamentous fungi, several distinct classes of photoreceptors have been identified, including the blue-light-responsive White collar regulatory complex and the red-light-responsive phytochromes (Herrera-Estrella and Horwitz, 2007). If the protein encoded by *PHL1* functions as a photoreceptor or exhibits a photosensory regulatory function, it may interact with other classes of photoreceptors to regulate cercosporin biosynthesis. Another possibility is that the protein encoded by *PHL1* functions either alone or in association with a regulatory complex to regulate signal transduction pathways that integrate inputs from multiple environmental stimuli. Future characterization of molecular mechanisms through which *PHL1* regulates secondary metabolism and fungal development should provide the answer to at least some of these questions.

In summary, we present evidence that the protein encoded by *PHL1* of *C. zeae-maydis* is an important component of photoreactivation after exposure to UV light and plays a minor role in other light-dependent processes, including conidiation and cercosporin biosynthesis. Our findings present the strongest evidence to date that fungal members of the cryptochrome/6–4 photolyase family exhibit photosensory regulatory functions. However, the exact

molecular function of *PHL1* is unclear; whether it functions as a photolyase, photoreceptor, or some combination of the two remains to be determined.

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References

- Alejandre-Durán, E., Roldán-Arjona, T., Ariza, R.R., Ruiz-Rubio, M., 2003. The photolyase gene from the plant pathogen *Fusarium oxysporum* f.sp. *lycopersici* is induced by visible light and agr-tomatine from tomato plant. *Fungal Genet. Biol.* 40, 159–165.
- Bernard-Pierrot, I., Delbe, J., Caruelle, D., Barritault, D., Courty, J., Milhiet, P.E., 2001. The lysine-rich C-terminal tail of heparin affix regulatory peptide is required for mitogenic and tumor formation activities. *J. Biol. Chem.* 276, 12228–12234.
- Berrocal-Tito, G., Sametz-Baron, L., Eichenberg, K., Horwitz, B.A., Herrera-Estrella, A., 1999. Rapid blue light regulation of a *Trichoderma harzianum* photolyase gene. *J. Biol. Chem.* 274, 14288–14294.
- Berrocal-Tito, G.M., Esquivel-Naranjo, E.U., Horwitz, B.A., Herrera-Estrella, A., 2007. *Trichoderma atroviride* PHR1, a fungal photolyase responsible for DNA repair, autoregulates its own photoinduction. *Eukaryot. Cell* 6, 1682–1692.
- Bluhm, B.H., Woloshuk, C.P., 2005. Amylopectin induces fumonisin B₁ production by *Fusarium verticillioides* during colonization of maize kernels. *Mol. Plant Microbe Interact.* 18, 1333–1339.
- Bluhm, B.H., Kim, H., Butchko, R.A.E., Woloshuk, C.P., 2008. Involvement of *ZFR1* of *Fusarium verticillioides* in kernel colonization and the regulation of *FST1*, a putative sugar transporter gene required for fumonisin biosynthesis on maize kernels. *Mol. Plant Pathol.* 9, 203–211.
- Casas-Flores, S., Rios-Momberg, M., Bibbins, M., Ponce-Noyola, P., Herrera-Estrella, A., 2004. BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*. *Microbiology–SGM* 150, 3561–3569.
- Chen, H., Lee, M.H., Daub, M.E., Chung, K.R., 2007. Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae*. *Mol. Microbiol.* 64, 755–770.
- Cooley, C., Bluhm, B.H., Reuhs, B.L., Woloshuk, C.P., 2005. Glass-fiber disks provide suitable medium to study polyol production and gene expression in *Eurotium rubrum*. *Mycologia* 97, 743–750.
- Daub, M.E., Ehrenshaft, M., 2000. The photoactivated *Cercospora* toxin cercosporin: contributions to plant disease and fundamental biology. *Annu. Rev. Phytopathol.* 38, 461–490.
- de Laat, W.L., Jaspers, N.G., Hoeijmakers, J.H., 1999. Molecular mechanism of nucleotide excision repair. *Genes Dev.* 13, 768–785.
- Douki, T., Cadet, J., 2001. Individual determination of the yield of the main UV-induced dimeric pyrimidine photoproducts in DNA suggests a high mutagenicity of CC photolesions. *Biochemistry* 40, 2495–2501.
- Essen, L.O., Klar, T., 2006. Light-driven DNA repair by photolyases. *Cell. Mol. Life Sci.* 63, 1266–1277.
- Felsenstein, J., 2008. Phylip 3.67. Available from: <<http://evolution.genetics.washington.edu/phylip.html>>.
- He, Q., Liu, Y., 2005. Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. *Genes Dev.* 19, 2888–2899.
- Herrera-Estrella, A., Horwitz, B.A., 2007. Looking through the eyes of fungi: molecular genetics of photoreception. *Mol. Microbiol.* 64, 5–15.
- Hishida, T., Iwasaki, H., Yagi, T., Shinagawa, H., 1999. Role of walker motif A of RuvB protein in promoting branch migration of Holliday junctions. *J. Biol. Chem.* 274, 25335–25342.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. ClustalW2 and ClustalX version 2. *Bioinformatics* 23, 2947–2948.
- Lin, C., Todo, T., 2005. The cryptochromes. *Genome Biol.* 6, 220.
- Pollock, S., Kozlov, G., Pelletier, M.-F., Trempe, J.-F., Jansen, G., Sitnikov, D., Bergeron, J.J.M., Gehring, K., Ekiel, I., Thomas, D.Y., 2004. Specific interaction of ERp57 and calnexin determined by NMR spectroscopy and an ER two-hybrid system. *EMBO J.* 23, 1020–1029.
- Proctor, R.H., Hohn, T.M., McCormick, S.P., 1997. Restoration of wild-type virulence to *Tri5* disruption mutants of *Gibberella zeae* via gene reversion and mutant complementation. *Microbiology* 143, 2583–2591.
- Sambrook, J., Russell, D.W., 2001. *Molecular Cloning: A Laboratory Manual*, third ed. Cold Spring Harbor Laboratory Press, New York.
- Shim, W.B., Dunkle, L.D., 2003. CZK3, a MAP kinase kinase homolog in *Cercospora zeae-maydis*, regulates cercosporin biosynthesis, fungal development, and pathogenesis. *Mol. Plant Microbe Interact.* 16, 760–768.
- Sinha, R.P., Hader, D.P., 2002. UV-induced DNA damage and repair: a review. *Photochem. Photobiol. Sci.* 1, 225–236.

- Tomkinson, A.E., Bardwell, A.J., Bardwell, L., Tappe, N.J., Friedberg, E.C., 1993. Yeast DNA repair and recombination proteins Rad1 and Rad10 constitute a single-stranded-DNA endonuclease. *Nature* 362, 860–862.
- Waterworth, W.M., Jiang, Q., West, C.E., Nikaido, M., Bray, C.M., 2002. Characterization of *Arabidopsis* photolyase enzymes and analysis of their role in protection from ultraviolet-B radiation. *J. Exp. Bot.* 53, 1005–1015.
- Weber, S., 2005. Light-driven enzymatic catalysis of DNA repair: a review of recent biophysical studies on photolyase. *Biochim. Biophys. Acta* 1707, 1–23.
- West, S.C., 1997. Processing of recombination intermediates by the RuvABC proteins. *Annu. Rev. Genet.* 31, 213–244.
- Yang, H.-Q., Wu, Y.-J., Tang, R.-H., Liu, D., Liu, Y., Cashmore, A., 2000. The C termini of *Arabidopsis* cryptochromes mediate a constitutive light response. *Cell* 103, 815–827.
- Yamazaki, S., Ogawa, T., 1972. The chemistry and stereochemistry of cercosporin. *Agric. Biol. Chem.* 36, 1707–1718.